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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) **High Level Recombinant Protein Production Using  
Conditional Helper-Free Adenovirus Vector**

(72) Massie, Bernard - Canada ;  
Langelier, Yves - Canada ;  
Lamarche, Nathalie - Canada ;

(73) Majesty (Her) In Right of Canada as Represented by the  
National Research Council of Canada - Canada ;

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ABSTRACT OF THE DISCLOSURE

The invention relates to a recombinant transfer vector for introducing a DNA sequence encoding a recombinant protein into an adenovirus genome. The transfer vector includes an expression cassette comprising sequentially a transcription promoter, a high efficiency leader, at least one splicing signal, an enhancer-like sequence, a cloning site and a plurality of polyadenylation sites.

TITLE OF THE INVENTION

High level recombinant protein production using conditional helper-free adenovirus vector.

FIELD OF THE INVENTION

5           The invention relates to adenovirus transfer vectors, particularly to transfer vectors allowing for the production of high levels of recombinant proteins.

BACKGROUND OF THE INVENTION

10           Biological production of proteins through recombinant DNA technology has been one of the leading aspects in biotechnology research over the last decade. To achieve economically viable levels of expression while still obtaining a biologically active protein, both eukaryotic and prokaryotic systems have been studied.

15           Ever since Cohen and Boyer first introduced foreign genes into bacterial strains by transformation, considerable emphasis has been placed on the use of bacterial systems for the expression of foreign proteins. The bacterial host of choice for the expression of  
20           heterologous genes has for a long time been *E. coli* for both practical and economical considerations. However, the insertion of cloned DNA sequences into an expression unit does not guarantee efficient gene expression when the expression unit is introduced into the bacterial host  
25           cell.

Hence, despite the versatility and efficacy of its expression vectors, with levels of expression in the

range of 20 to 50% of total cellular proteins, *E. coli* suffers several limitations for the expression of different categories of heterologous proteins especially those that undergo complex post-translational modifications such as many viral and mammalian proteins. Those limitations include inappropriate or lack of post-translational modifications, incorrect folding, proteolytic degradation, inefficient secretion and, recently reported, amino acid misincorporation.

Because of the limitations described above for *E. coli* expression systems, efforts have been directed towards the development of more sophisticated expression systems including other procaryotes, lower eucaryotes such as yeast and higher eucaryotes such as mammalian and insect cells. From the review of the amazingly vast literature reports on the expression of recombinant proteins appears to emerge the increasingly accepted notion that there is no "universal expression system". The current trends in the field is to tailor the development of expression systems to fit the specific expression needs. It is in that perspective that insect virus vectors and adenovirus vectors have been initially developed, mainly to exploit their respective capacity to express recombinant proteins in insect and human cells.

Baculoviruses and entomopoxviruses are widely known insect viruses that have been isolated from a large number of insect species in widespread geographical

locations. In recent years, baculovirus and poxvirus  
vectors have achieved widespread acceptance for their  
ability to express proteins of agricultural and medical  
importance. For example, a baculovirus vector was used  
5 to express the first recombinant HIV envelop proteins to  
receive FDA approval for clinical evaluation as a  
candidate vaccine for AIDS.

Poxvirus research, and more particularly the use  
of vaccinia virus, a prototypic member of the group of  
10 poxviruses, has led to eukaryotic cloning and expression  
of vectors useful in various biological and medical  
applications. In 1982, Panicali and Paoletti reported in  
Proc. Natl. Acad. Sci., Vol. 1979, pp. 4927-4931 (August  
1982) that endogenous subgenomic elements could be  
15 inserted into infectious progeny vaccinia virus via  
recombination *in vivo*. This ability to integrate vaccinia  
virus DNA sequences into infectious vaccinia virus progeny  
suggested the possibility for insertion of foreign genetic  
elements into infectious vaccinia virus via similar  
20 protocols. In order to test their assumption, Panicali  
and Paoletti inserted the herpes virus thymidine kinase  
(TK) gene into a number of vaccinia virus preparations and  
obtained pure cultures of recombinant vaccinia virus  
expressing the herpes virus gene.

25 It was reported that vaccinia virus appear to  
have several advantages over other eukaryotic vectors.  
Most noteworthy was the fact that virus infectivity was

not impaired by insertion and expression of foreign gene in contrast to defective SV40 and retrovirus vectors.

5       The vaccinia virus has been successfully used as an expression vector through the insertion of foreign genes into a non-essential region of the viral genome via homologous recombination. However, some drawbacks have also been associated with the use of this virus. The most difficult problem appears to reside in the fact that vaccinia expression vectors are not capable of producing  
10       abundant foreign proteins because of the absence of known strong promoters.

      Baculovirus vectors have also been used for the expression of foreign genes in insect cells. Indeed, in the case of baculovirus, two very strong and very late  
15       promoters are responsible for the expression of two extremely abundant proteins, polyhedrin and p10, which can together constitute as much as 50% of total cellular proteins in baculovirus-infected cells.

*Antographa californica* nucleopolyhedrosis virus  
20       (ACNPV) is the prototype virus of the family *Baculoviridae*. This virus has a wide host range and infects a large number of species of lepidopteran insects. During ACNPV infection, two forms of viral progeny are produced.

25       The first form consists of extracellular virus particles (ECV) that are responsible for dissemination of the virus within the infected host by either endocytosis

or fusion. The second form of viral progeny is an occluded virus particle (OV). These OV particles are imbedded in proteinaceous viral occlusions. The major structural protein forming the occlusion matrix is a polyhedrin protein having a molecular weight of 29,000 daltons.

These viral occlusions are an important part of the natural virus life cycle, providing the means for transmission of the virus from one host to another. They provide the virions, a degree of protection against external environmental factors that would otherwise rapidly inactivate the extracellular virus particles. The occlusions dissolve in the alkaline environment of the insect gut, releasing the virus that invades and replicates in the cells of the mid-gut tissue.

AcNPV possesses several properties that make this virus ideally suited as an expression vector for cloned eukaryotic genes. Since occlusion of the virus is not absolutely essential for viral growth, the polyhedrin gene provides a non-essential region for the AcNPV genome in which foreign DNA may be inserted. Placing foreign genes of interest under the control of either the polyhedrin or the p10 promoter have led in the best cases to production of recombinant proteins at 20-25% of total cellular proteins. The rapid construction of efficient transfer vectors has also been facilitated by the

relatively low complexity of gene regulation in the expression of the polyhedrin and p1C baculovirus genes.

Using the properties of AcSPV, a wide variety of eukaryotic and prokaryotic genes have been expressed successfully with baculovirus vectors in insect cells.

However, expression levels for different genes inserted into the same vector are often different and are related to the length and nature of the leader sequence proceeding the foreign gene. Even in the best available vectors, there is some variability in expression levels depending on factors such as the nature of the gene and the protein expressed.

Furthermore, careful characterization of numerous recombinant proteins has pointed to some problems in post-translational modifications in insect cells, such as impaired glycosylation, incomplete proteolytic cleavage of poly protein precursors, and inefficient secretion. This would appear to preclude the utilization of this expression system for the production of numerous complex mammalian proteins. In this regard, other alternatives better suited for the expression of mammalian proteins, such as adenovirus vectors, were also developed.

Adenoviruses (Ad) have first been isolated over three decades ago. Since then, many efforts have been invested into defining their biological properties. The intimate association that these viruses have with their host during infection has potentiated their value as tools



for exploring the mechanisms of macromolecular biosynthesis in mammalian cells.

The temporal pattern of adenovirus infection of human cells is generally demarcated into two phases of expression, early and late, which are separated by the onset of replication after about 8 hours. Early in infection, at least 7 promoters are active, generating transcripts from early regions 1-4. Over 30 messengers corresponding to the early regions have been identified by RNA analysis and/or cDNA cloning.

In contrast, the high levels of expression of the abundant viral late proteins are the result of the strong transcriptional activity of one promoter, the major late promoter (MLP) which is responsible for the production of some twenty late proteins encoded by an equivalent number of mRNA's. These mRNA's are all derived from one very long primary transcript by maturation processes involving differential splicing and polyadenylation events. Among those late proteins, three structural proteins, namely hexon (15-20% of total cellular proteins), fiber (8-10%), and penton (2-4%), and one non-structural protein named 100K (5-10%), constitute collectively as much as 35% of total cellular proteins in Ad-infected cells, whereas the remaining minor late proteins would constitute some 5%. Figure 1 shows an autoradiogram of the late structural proteins metabolically labelled with  $S^{35}$  methionine from adenovirus

infected 293 cells. The AdPyR39 recombinant was produced following the description provided by Massie et al. in 1986, Molecular and Cellular Biology, Vol. 6, No. 8, pp. 2872-2883, hereby incorporated by reference. The relative  
5 abundance of these late viral proteins can fluctuate depending on infection conditions. However, little is known about the mechanism which regulates this phenomenon. In any case, only a small portion of the structural proteins which are synthesized in copious amount, 20-30%  
10 of the hexon and 1-5% of penton and fiber respectively, are assembled into functional nucleocapsids. Therefore, it was soon realized that appropriate manipulations of Ad genome could potentially result in the construction of Ad recombinants expressing foreign proteins at very high  
15 levels.

The first human adenovirus (Ad) vectors have been developed in the early 1980's. These vectors have been used to express a wide variety of viral and cellular genes (for a complete review, see Berkler (1988),  
20 Biotechniques, Vol. 6, No. 7, pp. 616-629, hereby incorporated by reference). Currently, there are three potential commercial applications for Ad vectors, namely in 1) high level expression of heterologous proteins, 2) live viral sub-unit vaccines and 3) gene transfer vectors  
25 for establishing stable cell lines or gene therapy.

Adenovirus vectors appeared promising for expression of high levels of protein, since transcription

from the major late promoter was so efficient and high levels of translation were accompanied by host protein synthesis shut-off late in infection, facilitating protein isolation. Furthermore, human adenoviruses can replicate efficiently to very high titers ( $10^9 - 10^{10}$  pfu/ml) in human cells as well as other mammalian cells and adenoviruses produce their late proteins at levels that reach 30 to 40% of total cellular proteins. Finally, they can be propagated in suspension cultures thereby demonstrating a clear potential for large scale production.

However, because of the complexity in the regulation of gene expression in adenoviruses, the development of their full potential as high level expression vectors lagged behind baculovirus vectors. In fact, the majority of recombinant adenoviruses constructed thus far express only low to moderate levels of heterologous proteins. These levels are usually lower than the normal levels of adenovirus late proteins. Only a hand-full of examples of adenovirus recombinants were shown to express interesting levels of recombinant proteins when compared to some of the abundant adenoviral late proteins. Examples include AdSVR112 (Gluzman et al., 1982, Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory Press, N.Y., pp. 187-192) which expresses the SV40T antigen at 3.8% of total cellular proteins (see Simianis and Lane, 1985; see also Figure 1) and Ad5-RR2<sup>HSV</sup>

which expresses the HSV ribonucleotide reductase subunit 2 (R2) at 4.6% of total cellular protein (Lamarche et al., 1990, Journal of General Virology, 71, 1785-1792, hereby incorporated by reference).

5           Also described in the literature are adenovirus recombinant that appear to produce foreign proteins at levels which are somewhat between the level at which the 100 K protein is produced and the level at which the fiber protein is produced, although no accurate quantitation was  
10       reported in those latter cases. However, it seems that none of these Ad recombinant succeeded in expressing their heterologous protein at a level equivalent to or higher than the level of hexon or fiber which are respectively the first and second most abundant proteins in Ad-infected  
15       cells.

          A better understanding of the molecular mechanisms underlying the complex regulation of gene expression in adenoviruses is essential in order to construct transfer vectors which exploit the full  
20       potential for high level of expression of this system. One example among the best recombinant cistron assembled so far for high level of expression of foreign genes in adenovirus is represented in the transfer vector pAdBM1 (Lamarche et al. supra). In this vector, the expression  
25       cassette includes sequentially: a translational promoter (MLP), a high efficient translational leader (Ad2

tripartite leader) splicing signals, a cloning site, and multiple polyadenylation sites.

5           Leong et al. in (1990), *Journal of Virology*,  
Vol. 64, No. 1, pp. 51-60, reported that sequence-specific  
binding proteins are induced during the late phase of  
infection of adenovirus type 2 (Ad2). These proteins  
interact with 3 regions in the first intron of the major  
late promoter transcription unit from positions +37 to  
+68, +80 to +105 and +105 to +125 relative to the  
transcription initiation site. To measure the  
10       significance of these binding sites on transcription, the  
binding sites were deleted and it was found that these  
deletions caused significant reductions in the rate of  
transcription, specifically during the late phase of  
infection. The authors concluded that the results  
15       indicated that the high rate of transcription from the  
major late promoter during the late phase of infection  
resulted from the interaction of virus-induced  
transcription factors with 3 binding sites located in the  
intron between the first and the second active portions of  
20       the tripartite leader.

          In a recent article entitled "Assembly of  
enhancers promoters and splice signals to control  
expression of transferred genes" (1990), *Methods and*  
25       *Enzymology*, Vol. 185, pp. 512-527), Kriegler indicated  
that the most critical variable in the design of chimeric  
expression cistrons is the selection of an enhancer

element or elements for inclusion in the recombinant molecule. First identified in the genomes of SV40 and murine retroviruses, enhancers are described by Kriegler as being the most peculiar of all known expression elements. Kriegler mentions that the key properties that make an expression element an enhancer include 1) they are relatively large elements and may contain repeated sequences that can function independently, 2) they may act over considerable distances, up to several thousands base pairs, 3) they may function in either orientation, 4) they may function in a position independent manner and can be within or downstream of the transcribed region but can only function in cis (if several promoters lie near by, the enhancer may preferentially act on the closest) and 5) they may function in a cell type or tissue-specific manner.

An analysis of the effects of the variation of position of the SV40 enhancer on the expression of multiple transcription units in a single plasmid revealed two types of position effects. One position effect is called promoter occlusion and results in reduced transcription at a downstream promoter if transcription is initiated at a nearby upstream promoter. This effect does not involve enhancer elements directly, even though the effect is most pronounced when the downstream promoter lacks an enhancer element. The second effect stems from the ability of promoter sequences to reduce the effect of

a single enhancer element on other promoters in the same plasmid.

Thus, according to Kriegler, the SV40 enhancer element is a complex structure whose function is subject to some position effects and whose cell-type-specific activation is dependent, in part, on the absence or presence of active cellular factors or proximal sequences.

Another example given by Kriegler is one of a viral enhancer described in the hepatitis B virus. This enhancer is located 3' to the hepatitis B virus surface antigen coding sequences but is contained within the mature viral transcripts. Authors have reported that the HBV enhancer can dramatically increase expression levels of genes controlled by the SV40 enhancer/promoter but only when the enhancer is located within the transcribed region of the gene. Further, this effect appears to be orientation dependent, a violation of enhancer rules. Hence, the conclusion drawn by Kriegler on enhancers appears to be that enhancers are highly varied and function in a variety of ways. It therefore seemed difficult to predict where or how a so-called enhancer sequence may be displaced in the genome of a recombinant adenovirus vector to enhance expression levels.

The human Ad MLP is one of the strongest mammalian promoters known. Although active in the early phase of the infection, its transcriptional activity is increase 30-50 fold during the late phase. It has been

shown that a number of cis-acting sequences are essential to confer the full transcriptional activity to the MLP (see Mondésert and Kédinger, 1991, Nucleic Acids Research, vol. 19, No. 12, 3221-3228, hereby incorporated by reference). These include an upstream element (UE) between -67 and -49 relative to the transcriptional start site, a TATA box centered at -28, and an initiator element encompassing the transcription start site. In addition, some downstream element (DE) have been mapped and designated, R1 (+37 to +68), DE1 (+85 to +96) or R2 (+80 to +105), and DE2 (+109 to +124) or R3 (+105 to +125) (see Figure 2). While the UE, the TATA box and the R1 downstream element have been shown to be important for basal transcriptional activity of the MLP both at early and late times, the DE (DE1 and DE2) would be essential for late phase specific activation. DE1 and DE2 are functionally redundant and probably bind to the same transcription factor(s). They may also interact synergistically with the UE by an unknown mechanism, to bring about their late phase specific transcriptional activation. At this point, it is not clear whether these cis-acting sequences are "enhancer-like" or downstream promoter elements and whether enhanced expression could be obtained by inserting them in a transfer vector. These sequences are missing in all of the MLP currently used in Ad transfer vectors described so far. This appears to explain in part the limited success obtained with the



2053187

reported Ad recombinants. In any event, the inherent difficulty in properly evaluating the position at which enhancer-like or downstream promoter elements could be inserted to enhance expression still remains to be solved.

5 SUMMARY OF THE INVENTION

10 In accordance with the present invention, there is provided an adenovirus recombinant transfer vector to be used in the production of high levels of heterologous proteins in host cells. The high levels of expression are obtained through the use of at least one enhancer sequence, optionally conjugated to other enhancer sequences placed at specific regions on the adenovirus vector. Thus, the present invention relates to a recombinant transfer vector capable of introducing a DNA  
15 sequence encoding a recombinant protein into an adenovirus genome. The transfer vector includes an expression cassette comprising a cloning vehicle having a DNA sequence comprising sequentially a transcription promoter, a high efficiency leader, at least one splicing signal, an  
20 enhancer-like sequence, a cloning site and a plurality of polyadenylation sites. The enhancer-like sequence is to be located between the high efficiency leader and the cloning site where the foreign gene is to be incorporated. Preferably, the enhancer-like sequence corresponds to the  
25 +30 to +130 AD2MLP sequence described in 1990, Journal of Virology, Vol. 64, No. 1, pp. 51-60, the contents of which is hereby incorporated by reference. In another preferred

2953187

aspect of the present invention, the adenovirus vector may also include another enhancer-like sequence to be located immediately upstream from the transcription promoter.

5       The invention also relates to a recombinant adenovirus expression vector capable of expressing a DNA sequence encoding a recombinant protein in mammalian cells, preferably in human cells. The expression vector is an adenovirus genome comprising a DNA sequence encoding a recombinant protein and a DNA sequence comprising  
10       sequentially a transcription promoter, a high efficiency leader, at least one splicing signal, an enhancer-like sequence, a cloning site and a plurality of polyadenylation sites.

      Also within the scope of the present invention  
15       is a method for producing a recombinant adenovirus expression vector possessing the ability to express a DNA sequence encoding a recombinant protein in mammalian cells. The method comprises cleaving adenovirus DNA to produce a DNA fragment comprising sequentially a  
20       transcription promoter, a high efficiency leader, at least one splicing signal, an enhancer-like sequence, a cloning site and one or a plurality of polyadenylation sites; preparing a recombinant transfer vector by inserting the DNA fragment into a cloning vehicle and thereafter  
25       inserting at least one DNA sequence encoding a recombinant protein into the thus modified cloning vehicle such that the DNA sequence encoding the heterologous protein is

under the control of the transcription promoter;  
contacting the recombinant transfer vector with adenovirus  
DNA through homologous recombination; and isolating and  
recovering the desired recombinant adenovirus expression  
5 vector.

Also within the scope of the present invention  
is a method for synthesizing a recombinant protein which  
comprises infecting mammalian host cells with a  
recombinant adenovirus expression vector wherein the  
10 expression vector is an adenovirus genome comprising a DNA  
sequence encoding a recombinant protein and a DNA sequence  
comprising sequentially a transcription promoter, a high  
efficiency leader, at least one splicing signal, an  
enhancer-like sequence, a cloning site and a plurality of  
15 polyadenylation sites; growing the mammalian host cells  
and recovering the desired product.

With the construction of the adenovirus  
expression transfer vector of the present invention,  
unprecedented levels of recombinant gene expression have  
20 been achieved in preferred host cells such as human and  
mammalian cells. More preferably, human 293 cells  
infected with helper-free adenovirus recombinants  
generated with the best mode of the transfer vector of the  
present invention have produced recombinant proteins that  
25 represent the most abundant polypeptide in the cell, even  
exceeding the level of the most abundant viral late  
protein, the hexon.

With the transfer vector of the present invention, commercial production of recombinant proteins from host cells infected with recombinant plasmids generated from this transfer vector is rendered possible.

5 One major factor for the economical production of recombinant proteins in the adenovirus system lies in the possibility to produce recombinant proteins in suspension cultures. For example, the human 293, human K562 and the  
10 Hela cell lines or derivatives thereof bearing the adenovirus E1 region which have been adapted to grow as suspensions can be used preferentially. High levels of expression can also be achieved in other types of mammalian and human cells, but it is to be note that the use of helpers may in some instances be necessary. In  
15 order to achieve these exceptional and economical viable levels of expression, it appears that the position in the transfer vector of the enhancer-like sequence, preferably the Ad2MLT enhancer-like sequence referred to previously is critical. The present invention will be more readily  
20 illustrated by referring to the following description.

IN THE DRAWINGS

Fig. 1 Relative quantitation of protein synthesis in 293 cells infected by various adenovirus recombinants. 293 cells were infected with Ad5ΔE1/d1309, Ad5pYR39 or  
25 AdSVR112. At 20 h after infection, they were labeled for 2 h with [<sup>35</sup>] methionine. Total proteins were extracted, resolved by SDS-PAGE electrophoresis (10%), and revealed by

autoradiography. The position of the Ad abundant late proteins, hexon, 100K, penton and fiber as well as recombinant SV40 large T antigen, are indicated.

Fig. 2 Diagrams of Ad5 endogenous MLP DNA fragment as found in its normal location in the genome or ectopic MLP DNA fragments as found in Ad transfer vectors. (See text for details). The diagrams are not drawn to scale. Symbols: (SS) splicing signal, (tpl) tripartite leader, (pa) polyadenylation signal.

Fig. 3 Production of recombinant Ad.

Fig. 4 Complete genetic map of pAdBM1 and pAdBM5 transfer vectors. As illustrated on the diagrams, pAdBM5 was derived from pAdBM1 by successive cloning of BKV enhancer elements at Bgl II sites nucleotides 380 and 2590, and Ad2 MLP enhancer-like element at Bbl II site nucleotide 2010 on pAdBM1 map. All of the genetic elements present in pAdBM1 have been described in details in Lamarche et al. supra. The inner number on the vector refer to position map units (m.u.) on Ad5 genome (for symbols, see legend, Fig. 2). Briefly, pML2 is the E. coli replicon, the segments with dashed lines (0-1, and 9.4-15.5 m.u.) bracketing the expression cassette (between 1.0 and 9.4 m.u.), are Ad5 subgenomic portions involved homologous recombination to generate Ad recombinant as shown in Fig. 3 and in Ad replication.

Fig. 5 Relative quantitation of HSV R1 and R2 produced in  
helper-free Ad5 recombinants derived from pAdBM1 or pAdBM5  
transfer vectors. 293 cells were infected with Ad5-  
ΔE1/ΔE3, indicated in panels A and B. Mock 293 are non-  
infected 293 cells. At 48 h after infection, total cell  
extracts were prepared and proteins were resolved by 8%  
SDS-PAGE (panel A) or 10% SDS-PAGE (panel B). The  
resolved proteins were detected by staining with Coomassie  
blue. Molecular weight markers are shown on the left in  
panel A and on the right in panel B. The position of the  
abundant late proteins, hexon, 100K, fiber as well as the  
recombinant R1 and R2, are indicated.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to an adenovirus transfer  
vector useful in achieving production of high levels of  
recombinant proteins in mammalian cells. The expression  
cassette of the transfer vector of the present invention  
includes a fragment which comprises sequentially a  
transcriptional promoter, a high efficiency leader, at  
least one splicing signal, a first enhancer-like sequence,  
a cloning site, and a plurality of polyadenylation sites.  
Preferably, the sequence also comprises a second enhancer-  
like sequence upstream from the transcriptional promoter  
and two 5' donor sites located upstream of the MLP  
enhancer-like sequence and one 3' acceptor site located  
immediately downstream. The presence of the second  
enhancer-like sequence appears to be optional as marginal

enhancement in expression is obtained when it is present in the vector without the first enhancing-like sequence.

5 Preferably, the transfer vector of the present invention lacks the E3 coding region which is known to be dispensible for growth of the virus in cell cultures as well as the E1 coding region which encodes proteins essential for the activation of adenovirus promoters. The loss of the E1 region in the vector is complemented by an appropriate mammalian cell line such as 293 cells which  
10 constitutively express the E1 protein from an integrated E1 (preferably Ad5E1) coding region. These deletions allow for the insertion of up to a 7-8 kb of foreign DNA generating conditioner helper-free adenovirus vector. Adenovirus vectors are preferably suited for the  
15 expression of recombinant mammalian proteins, particularly but not exclusively of human origin.

Helper-free adenovirus recombinants generated with the transfer vector of the present invention are chosen to infect host cells in an appropriate medium.  
20 Preferably, the culture host cell is a mammalian host cell and more preferably, a human cell of the type described above.

Among the transcriptional promoters that may be used to prepare the transfer vector of the present  
25 invention, strong viral promoters are preferred because of their efficiency in directing transcription. However, other transcriptional promoters such as the mouse

metallothionein (MT-1) promoter, the SV40 late promoter, the SV40 early promoter and cytomegalovirus (CMV) promoter may be used. A particularly preferred viral promoter is the major late promoter (MLP) from adenovirus.

5           The transfer vector of the present invention also includes a high efficiently leader immediately downstream from the transcriptional promoter. Preferred leader sequences are viral leader sequences which include the adenovirus first leader and the adenovirus LI-IX  
10 leader, the SV40 leader and the parvovirus leader. A particularly preferred leader sequence is the high efficiency viral leader, adenovirus tripartite leader (TPL).

15           The presence of efficient splicing sequences (5' and 3') that have been functionally shown to participate in an mRNA splicing event may also be required. Preferred splicing sequences include 5' donor sites of either the first or the third segment of the tripartite leader and 3' acceptor site from an immunoglobulin gene.

20           With regard to the polyadenylation sites, it seems that a plurality of sites is preferred in order to generate the highest possible level of the appropriate recombinant proteins. The number of sites may vary from 2 to 5 but it would appear that the preferred number of  
25 sites be 3. The polyadenylation sites that may be chosen for use in the transfer vector of the present invention may be selected from but are not restricted to the group



consisting of SV40 early or late poly A signals, polyoma early poly A, Ad5 hexon mRNA poly A signals and  $\beta$ -globin poly A signal.

5       The preferred enhancer-like sequence to be introduced in the transfer vector of the present invention is a sequence normally located in the intron between the first and the second active sequence of the tripartite leader. In order to preserve the integrity of the tripartite leader, the enhancer-like sequence was  
10       relocated further downstream from its normal position. Preferably, the enhancer-like sequence is positioned about 303 nucleotides further downstream from its normal position in the Ad5 genome. This relocation of the enhancer-like sequence relative to its normal position in  
15       the Ad5 genome resulted in a 2.5 to 3 fold increase in recombinant protein expression when comparing a vector devoid of the enhancer-like sequence such as pAdBM1 with a vector bearing the enhancer-like sequence downstream from the tripartite leader such as pAdBM5. The enhancer-  
20       like sequence is specifically exemplified at page 54 of Leong et al. (Journal of Virology, 1990, Vol. 64, No. 1, pp. 51-60, hereby incorporated by reference). It is possible that other enhancer-like sequences may be used at a similar position. It is also preferred to use a second  
25       enhancer-like sequence immediately upstream of the translation promoter. A preferred enhancer sequence is the BKV Dun enhancer sequence which is described in Berg

et al., Nucleic Acids Research, Vol. 16, No. 18, 1988, pp. 9057, hereby incorporated by reference. Tests have been conducted using an enhancer sequence upstream from the promoter without an enhancing sequence between the tripartite leader and the cloning site without noticing substantial improvement in expression yields. This may be an indication that the second enhancer-like sequence located upstream from the promoter is not absolutely required but does not exclude the possibility of a synergistic interaction between the two enhancer-like sequences.

The transfer vector of the present invention has wide application in the construction of recombinant adenoviruses for the production of recombinant proteins in mammalian cells. These applications include the production of commercial quantities of therapeutic and commercially important proteins. The vectors of the invention may be adapted to include sequences encoding proteins of interest and coding sequences which enhance or enable the expression of biologically active proteins of interest. It is important to note that the transcription of the foreign gene in the resulting recombinant adenovirus takes place in the opposite orientation from the overall direction of transcription of the late adenovirus genome. This is required because it has been shown previously in other adenovirus recombinants that expression in the same orientation as the overall

direction of transcription can interfere with the normal expression of other adenoviral genes in the downstream E1b region.

5       The expression vectors of the present invention  
may be introduced into host cells by infection using  
methods described in the prior art. An example is the  
method described by Lamarche et al. supra. Various  
mammalian host cells can be used in the context of the  
present invention. Examples of mammalian cells include  
10   human cells and bovine cells. Suitable mammalian cell  
lines include the 293 (ATCC 1573) cell line, or HeLa and  
K562 cell lines as well as derivatives expressing AdE1 and  
isolates of these cell lines, although it will be obvious  
to those skilled in the art that other cell lines may be  
15   preferred for the production of particular proteins. The  
host cell line should be selected on the basis of its  
ability to produce the protein of interest at a high level  
and/or its suitability for very specific post-translational  
modification of the desired protein.

20       It was found that when using the best mode of  
the transfer vector of the present invention the  
recombinant adenovirus replicated efficiently to levels  
close to  $5 \times 10^9$  pfu/ml. It is to be noted that excessive  
expression levels may in some instances reduce the titer  
25   below acceptable levels. However, in the case of Ad5HSV-  
R1 and Ad5HSV-R2 recombinants that illustrate preferred  
embodiments of the present invention, even though a

reduction in titer was observed as a result of very high expression levels of the recombinant proteins, their respective titer was still routinely above  $5 \times 10^7$  pfu/ml, a level which is required for large scale production.

5 Indeed, these two recombinant proteins have been efficiently produced in 293 suspension cultures in volumes of up to 5 liters. Hence, the expression obtained with the preferred vectors of the present invention is likely to be close to the upper limit of the system when bearing  
10 scale-up considerations in mind.

The following example is provided to illustrate rather than limit the scope of the present invention.

Example 1.

15 Production of recombinant adenovirus.

The coding region of the desired heterologous gene is first cloned in a transfer vector such as pAdBM1 (or its derivatives) at the unique *Bam*HI cloning site, downstream of the strong Ad2 major late promoter. The  
20 construction of vector pAdMB1 is described in Lamarche et al. supra. Figure 3 demonstrates the production of recombinant adenovirus based on transfer vector pAdBM1.

The resulting recombinant plasmid is then rescued into the genome of the adenovirus vector  
25 Ad5ΔE1/ΔE3 by *in vivo* homologous recombination between overlapping sequences on the linearized plasmid and the large right-end fragment of the Ad5 genome, upon

2053187

5 cotransfection of human 293 cells. This cell line constitutively expresses the Ad5 E1 gene products which are essential for the helper-free propagation of Ad5ΔE1/ΔE3 derived recombinants. Digestion of Ad5ΔE1/ΔE3 viral DNA with Cla I prior to transfection allows for obtention of recombinant adenovirus at a frequency of 5-20%.

Construction of adenovirus recombinant.

10 1. The gene to express is first cloned in a transfer vector such as pAdBM-1 at the unique BamHI cloning site. The resulting recombinant plasmid is then rescued into the genome of the adenovirus vector Ad5ΔE1/ΔE3 by in vivo homologous recombination between overlapping sequences on the linearized plasmid and the large right-end fragment of the Ad5 genome.

15 2. Preparation of pAdBM1 transfer plasmid.

20 20-50 μg of plasmid DNA is digested with ClaI or EcoRI, extracted once with buffer-saturated phenol and chloroform/isoamyl alcohol (24:1), precipitated with 2.4 volumes of ethanol and resuspended in 50 μl of sterile TE 1/10. The concentration of the linearized plasmid is then estimated in an agarose gel.

3. Preparation of Ad5ΔE1/ΔE3 viral DNA.

25 1. 293 cells infected with Ad5ΔE1/ΔE3 at a m.o.i. of 5-10 are harvested 40-48 h post-infection, washed twice with cold PBS and resuspended in 10 mM Tris pH 7.9 (2 volumes per volume of cell pellet).

2. The cell pellet is then freeze-thawed 3 times to release intracellular viral particles, and extracted with 1,1,2-trichlorotrifluoroethane (freon) as follow: (all steps on ice). Mix an equal volume of cell suspension and freon and blend in an omnimixer at full speed for 2 min. Spin at 2K for 15 min., collect top phase (aqueous) and reextract the freon phase twice with the same volume of buffer (10 mM Tris pH 7.9).

3. The virions are purified through 2 consecutive CsCl gradients.

a) Step gradient

In SW 27 cellulose nitrate tubes, pour 8 ml of CsCl 1.4 (53 gr + 87 ml of 10 mM Tris pH 7.9) and very gently on top pour 56 ml of CsCl 1.2 (26.8 gr + 92 ml of 10 mM Tris pH 7.9). The aqueous phase containing the virions is then loaded on top of the discontinuous gradient (up to 22 ml/tube).

Centrifuge at 23K for 90 min at 0°C.

Collect the virus band by side puncture. Dilute 1/2 in 50 mM Tris pH 7.5, 1 mM EDTA.

b) Continuous gradient

Using a gradient maker, pour continuous CsCl gradient in SW27 cellulose nitrate tubes using 12 ml of CsCl 1.4 and 14 ml of CsCl 1.2. Load 8-10 ml of the diluted virus suspension on top very slowly.

Centrifuge at 23k for 16-20 hrs at 0°C.

Collect the virus band by side puncture and dialize against 100 volumes of 10 mM Tris pH 7.9, 1 mM EDTA (3 changes) and finally against 100 mM Tris pH 8.5 1 mM EDTA.

5 c) Purification of viral DNA from virions

Incubate at 37°C for 2 hrs with self-digested pronase at a final concentration of 1 mg/ml and SDS 0.5%.

10 Add NaCl to a final concentration of 100 mM, extract twice with buffer-saturated phenol, one with chloroform/isoamyl alcohol (24:1) and precipitate with 2.5 volumes of ethanol.

15 4. Ad5ΔE1/ΔE3 is digested with an excess of ClaI (map unit 10) to minimize the fraction of undigested viral genomes since they represent the background noise in the screening of recombinant viruses.

Typically, 5 units of ClaI/μg of viral DNA are added for 3 consecutive incubations of 1 hr at 37°C.

20 An aliquot of the preparative digestion is further digested with HindIII and analyzed by comparing the restriction pattern with the pattern obtained upon digestion with HindIII alone. If the ClaI digestion is incomplete, repeat the preceding step.

25 ClaI-cut Ad5ΔE1/ΔE3 DNA is then extracted once with buffer-saturated phenol and chloroform/isoamyl alcohol (24:1), precipitated with 2.5 volumes of ethanol and resuspended in 50 μl of sterile TE 1/10. The concentration is estimated in an agarose gel. Figure 4

illustrates the maps of both recombinant transfer vectors pAdBM1 and pAdBM5.

4. Transfection of 293 cells to generate A45 recombinants.

- 5 1. 293 are plated in 60 mm-diameter dishes at  $1.0 \times 10^6$  cells/ml one day prior to transfection (in DMEM + 10% FBS + antibiotics).
2. 5  $\mu$ g of transfer plasmid (linearized with *Cla*I or *Eco*RI) containing the gene to express is mixed with 5  
10  $\mu$ g of *Cla*I-cut Ad5 $\Delta$ E1/ $\Delta$ E3 viral DNA and transfected onto sub-confluent 293 cells using the standard calcium phosphate technique.
3. As a control for the transfection, 1  $\mu$ g of  
15 Ad5 $\Delta$ E1/ $\Delta$ E3 viral DNA + 9  $\mu$ g of carrier DNA is also transfected. This should yield more than 100 plaques.
4. After overnight incubation, the DNA-calcium phosphate co-precipitate is removed, the cells monolayer washed once with EGTA 1 mM in PBS and twice with PBS and splitted into 3 X 60 mm-diameter dishes.
- 20 5. After 4-6 hrs, the medium is removed and the cell monolayers are overlayed with Seaplaque agarose 1% (mix agarose 5% 1:5 with DMEM + 10% FBS + antibiotics).
6. Viral plaques (usually 20-60) that appear  
25 between days 5 and 15 post-transfection are picked (as agarose plugs) and grown on 293 cells into 24 wells plates ( $5 \times 10^4$  cells/well). (Complete CPE is obtained in 3-7 days).



5. Screening and purification of Ad5 recombinants.

1. Viral DNA from 200  $\mu$ l of lysate for each individual plaque is extracted with 10  $\mu$ l of 10% SDS and 10  $\mu$ l of pronase (20 mg/ml) incubated at 37°C for 2 hrs.

5 2. The DNA is then denatured by adding 40  $\mu$ l of 1M NaOH (incubated at R.T. for -10 min.) and neutralized by adding sequentially 40  $\mu$ l of 1M Tris pH 7.5 and 40  $\mu$ l of 1M HCl.

10 3. The DNA is finally put on a hybridization membrane using a dot blot apparatus and screen with the appropriate probe following standard procedures.

4. Positive plaque isolates are tested for expression and 2 of the best clones are further plaque purified twice.

15 6. Plaque assay.

1. The day before, 293 cells are seeded at  $5 \times 10^5$  cells/60 mm plate).

2. Viral lysates are diluted into completed medium up to  $10^{-7}$  and the last 3 dilutions ( $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ) are  
20 used to infect 293 cells (0.5 ml /60 mm plate, for 60-90 min).

3. The diluted lysates are removed and the monolayers overlaid with 5 ml of agarose 1% (mix agarose 4:5 with DMEM + 10% FBS + antibiotics). Plaques appeared  
25 within 5-10 days.

A novel Ad expression transfer vector, pAdBM5, that allows for the production of unprecedented levels of

recombinant protein using the Ad expression system has been described. High levels of expression were obtained by relocating the enhancer-like sequence in the intron located between active sequences 1 and 2 of the tripartite leader, as shown in Figure 2. It was shown that in human 293 cells infected with helper-free Ad recombinants generated with pAdBM5 transfer vector, the recombinant protein represents the most abundant polypeptide, even exceeding the level of the most abundant viral late protein, the hexon. Figure 5a illustrates the enhanced production of the recombinant protein in pAdBM5.

Furthermore, it seems that the actual level of expression obtained with pAdBM5 derived recombinants Ad is probably very close to the upper limit of the system. First, from the level obtained with the previously reported transfer vector pAdBM1 (Lamarche et al. supra) in the range of 4% of total cellular proteins, for both HSV R1 and R2 genes, up to 3 fold increase has been observed leading to levels as high as 20% of total cellular proteins, as shown in Figures 5a and b. This level is higher than the maximum obtained for the hexon (16%, see Figure 1) in 293 cells infected with Ad5 $\Delta$ E3 virus that do not express foreign protein and suggests that in pAdBM5 derived Ad recombinant the very high production of the recombinant protein takes place at the expense of the Ad abundant late proteins. Consistent with this interpretation is the observation that the titers of the

2053187

recombinant viruses are significantly reduced, although not to the extent that the large scale production of recombinant protein with the Ad expression system is affected.

5     Construction of lacI-R2 fusion protein vector

The HSV2-R2 coding region was expressed under the control of the lac promoter as a fusion protein containing 6 additional amino acid residues derived from the lacZ gene using the following cloning strategy:

- 10     1. A 2.7 kb (Bgl II-Pst I) fragment containing the HSV2-R2 coding region was first cloned into the polylinker site of pUC8 between the Bam HI and Pst I sites.
- 15     2. The 560 bp (EcoR I-Bam HI) fragment at the 5' end of the R2 gene was deleted and the vector self-ligated with the appropriate Bam HI linker to yield pMO2. The plasmid is shown in Figure 6.

Construction of vectors for authentic R2 expression

In order to produce large amount of authentic R2 protein, the R2 coding region was inserted into 3 expression vectors as depicted:

- 20     1. pGEMtac, an *E. coli* expression vector using the strong tac promoter. 2 constructions were generated in which the spacing between the Shine-Dalgarno motif and the R2 initiator ATG was 8 bp (Bgl II-Bcl I) or 13 bp (Bcl I-Bcl I) respectively.
- 25     2. pAc373I, an optimized baculovirus transfer vector, derived from pAc373, which uses the strong polyhedrin

2053187

promoter and contains the complete polyhedrin leader sequence upstream of the unique Bam HI cloning site. To generate recombinant baculovirus, pAc3731-R2 was rescued into the genome of *Autographa californica* nuclear polyhedrosis virus by *in vivo* homologous recombination upon cotransfection with wild type AcNPV DNA into S19 insect cells.

3. pAdBM1, an adenovirus transfer vector which uses the strong Ad2 major late promoter (MLP) and contains the adenovirus tripartite leader and a splice junction upstream of the unique Bam HI cloning site. To generate recombinant adenovirus, pAdBM1-R2 was rescued into the genome of the adenovirus vector Ad5ΔE1/ΔE3 by *in vivo* homologous recombination between overlapping sequences on the linearized plasmid and the large right-end fragment of the Ad5 genome, upon cotransfection of human 293 cells. A similar procedure was repeated to yield pAdBM5-R2. Figure 7 illustrates pGEMtac-R2, pAc3731-R2 and pAdBM1-R2 (pAdBM5-R2 is now shown).

#### R2 recombinant protein production and extraction

##### *E. coli*

*E. coli* (strain JA221) containing the R2 expression plasmids pMD2 or pGentac-R2 were grown to an OD<sub>590</sub> of 2.0. Protein extracts were prepared essentially as described by Ingemarson et al. in 1989, Journal of Virology, 63, pp.1 3769-3776, hereby incorporated by reference. Briefly, the bacteria were washed in 25 mM

HEPES (pH 7.6), resuspended in the same buffer to an OD<sub>590</sub> of 200, than freeze in liquid nitrogen and thawed on ice. KCl, PMSF and egg white lysozyme were added to final concentrations of 80 mM, 1 mM and 300 µg/ml, respectively, and the mixture was incubated on ice for 20 min. After another cycle of freezing and thawing, cell debris were removed by centrifugation at 44,000 g for 60 min at 4°C. The major part of the supernatant (crude extract) was frozen until further purification. A minor fraction was passed onto a small Sephadex G-25 column to remove RR inhibitory molecules and analysed for R2 reductase activity.

#### Adenovirus

Subconfluent 293 cells ( $1.0 \times 10^8$ ) in 850 cm<sup>2</sup> roller bottle were infected with Ad5BM1R2 or Ad5BM5R2 at 10 PFU/cells. The cells were harvested, usually 48 h., p.i., washed twice with PBS, pelleted and resuspended in 50 mM HEPES (pH 7.8) with cold 2 mM OTT. Proteins were extracted by sonication and insoluble materials were removed by centrifugation at 12,000 g for 10 min. A fraction of this crude extract (G-25-treated) was save to measure R2 activity and the remainder further purified.

#### Baculovirus

Sf9 cells were grown in suspension at a density of  $2 \times 10^6$  cells/ml and infected with the recombinant baculovirus BacR2 at 10 PFU/cell. 48 h., p.i., cells were

harvested and protein extracts were prepared as described above for the recombinant adenoviruses.

Purification of R2 protein

5       The first step of purification consists of 2  
successive salt precipitations. First, streptomycin  
sulfate was added to the crude extract to a final  
concentration of 1%. After 1 h. of stirring at 4°C, the  
suspension was centrifuged at 12,000 g for 20 min to  
remove the precipitated nucleic acids. Then, ammonium  
10       sulfate was added to obtain 30% of saturation for the *E.*  
*coli* extracts and 60% for the eukaryotic cell extracts.  
After 30 min of stirring at 4°C and centrifugation as  
above, the pellet was dissolved in 50 mM HEPES (pH 7.8),  
2 mM DTT (buffer A) and dialysed against the same buffer.  
15       As second step, an anion exchange chromatography  
was performed essentially as described by Lankinen et al.  
in Journal of General Virology, 1991, Vol. 12, pp. 1383-  
1392, hereby incorporated by reference. Briefly, after  
the ammonium sulfate precipitation, the supernatant was  
20       dialyzed overnight against two changes of 20 mM BisTris-  
HCl, pH 5.8 and 10% glycerol. The precipitate was removed  
by centrifugation at 24,000 g for 40 min at 4°C. The  
supernatant was then loaded onto an FPLC anion exchanger  
MonoQ hr 10/10 column (Pharmacia) and R2 protein was  
25       eluted with a gradient of KCl. Fractions containing the  
R2 protein were concentrated by ultrafiltration using  
Centriprep-10 (Amicon) and washed twice with buffer B

containing 10% glycerol. Protein R2 concentration in the purified fractions was measured by the Coomassie blue method of Bradford described in Anal. Biochem., 1976, Vol. 72, p. 248, hereby incorporated by reference and with an  $E_{280-310}$  of 52,000  $M^{-1}cm^{-1}$  for the nearly homogeneous preparations.

HSV R2 protein produced by the four expression vectors referred to previously is shown in Figure 8. The proteins present in the crude extracts (lanes 1, 4 and 9), after the ammonium sulfate precipitation (lanes 2, 5, 7 and 10) and after the FPLC column (lanes 3, 6, 8, 11 and 12) were separated by SDS-PAGE and the gel was stained by Coomassie blue. The total amount of protein loaded in each track are given in  $\mu g$  at the top of the figure and also the concentration of R2 protein expressed as percentage of the total amount of protein. Results are shown in Figure 8 and in Table 1.

#### R2 reductase assay

R2 reductase activity was determined in the presence of excess amounts of the HSV2 R1 subunit extracted from 293 cells infected with a recombinant adenovirus expression vector (R1 specific activity, 100 U/mg). Reductase activity was measured by monitoring the reduction of [ $^3H$ ]CDP as previously reported in Lamarche et al. supra. The standard reaction mixture contained 50 mM HEPES, pH 7.8, 4 mM NaF, 50 mM DTT, 50  $\mu M$  CDP, and 0.25  $\mu Ci$  of [ $^3H$ ]CDP. One unit of ribonucleotide reductase was

defined as the amount of enzyme subunit generating 1 nmol of dCTP/h under the standard assay condition.

5       The main conclusion of the present work is that the HSV-2 R2 protein produced by the two eukaryotic systems is 3 fold more active than the protein produced by *E. coli*. This is particularly striking from the comparison of the mean values obtained from six different ammonium sulfate preparations of pMD2 R2 (3,230 U/mg of R2) and of Ad5BM5 R2 (10,750 U/mg of R2). Preliminary  
10      results on the measurement of reductase activity of authentic R2 protein produced in *E. coli* by our pGEMtac vector indicated that this protein exhibits an activity similar to the one of the R2 fusion protein. Therefore, it appears unlikely that the presence of the extra 6 amino  
15      acids on this protein is responsible for its lower activity. Inefficiency of *E. coli* for the generation of the free tyrosil radical or some important post-traductional modifications could account for the lower activity.

20       The results also showed that the bacterial system with its high level of expression facilitate the purification of R2 protein: nearly homogeneous preparations can easily be obtained by a 2-step procedure. When applied to the adenovirus recombinant R2, the same  
25      procedure yielded preparations that were enriched to only 50-60% with pAdBM1 derived recombinants but to over 95% with pAdBM5 due to its higher production levels.



Table 1. COMPARISON OF EXPRESSION AND PURIFICATION OF HSV2 R2  
PROTEIN PRODUCED BY OUR DIFFERENT EXPRESSION VECTORS

	Ad5BM1R2- infected 293 cells	Ad5BM5R2- infected 293 cells	BacR2-infected Sf9 cells	<i>E. coli</i> (JA221)- pMD2	HSV-2-infected BHK cells
Expression level (%)	4.6	11	~2%	21	0.5
Degree of purity (%)	52	~50	36	99.5	N.D.
Specific activity (U/mg of R2)	9916	N.D.	9444	3442	9820

a. This % was estimated from the % measured after ammonium sulfate precipitation

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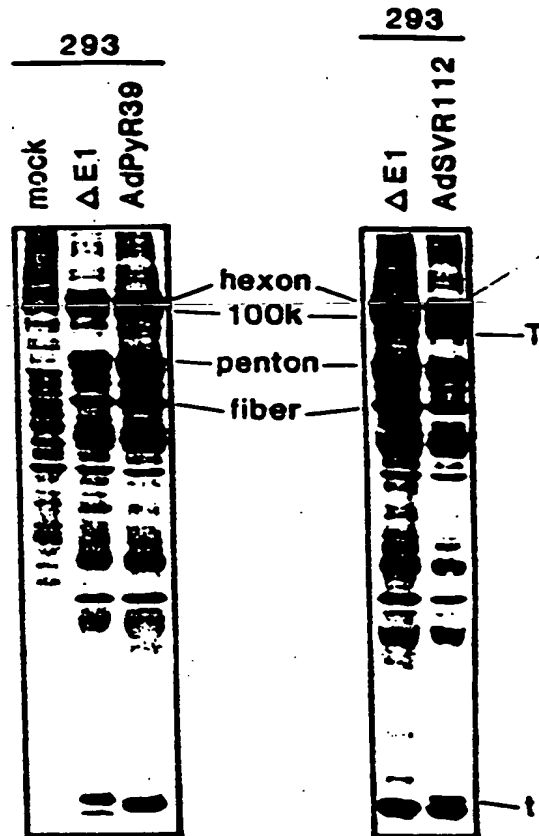
CLAIMS:

1. A recombinant transfer vector for introducing a DNA sequence encoding a recombinant protein into an adenovirus genome, said transfer vector including an expression cassette comprising sequentially a transcription promoter, a high efficiency leader, at least one splicing signal, an enhancer-like sequence, a cloning site and a plurality of polyadenylation sites.
2. An adenovirus transfer vector having the structure of vector pAdBM5 shown in Figure 4.
3. A recombinant adenovirus expression vector capable of expressing a DNA sequence encoding a recombinant protein in mammalian cells, said expression vector being an adenovirus genome comprising a DNA sequence encoding a recombinant protein and a DNA sequence comprising sequentially a transcription promoter, a high efficiency leader, at least one splicing signal, an enhancer-like sequence, a cloning site and a plurality of polyadenylation sites.

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Fig 1

Fig 1



Légende à lire : Auto radiogramme  
 protéines marquées in vitro 53T

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Ad5

MLP

Fig 2.

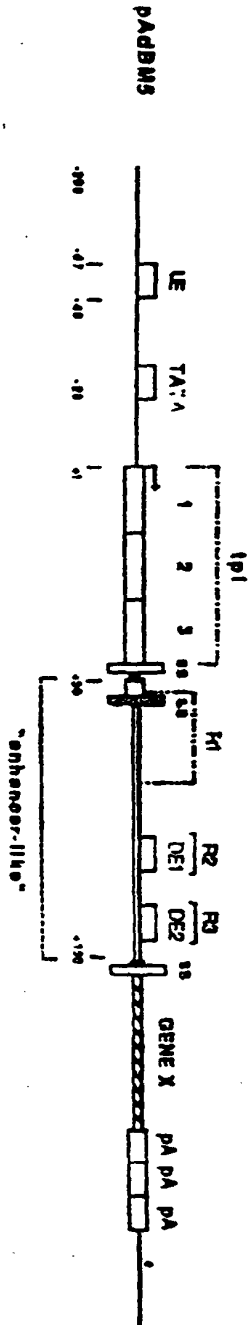
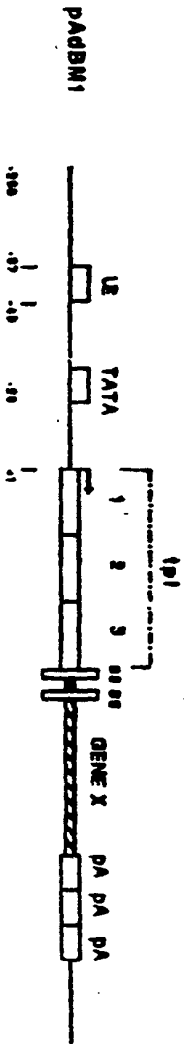
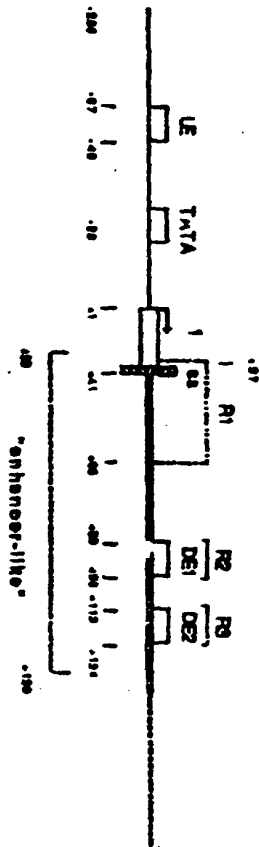


Fig 2



Fig 4

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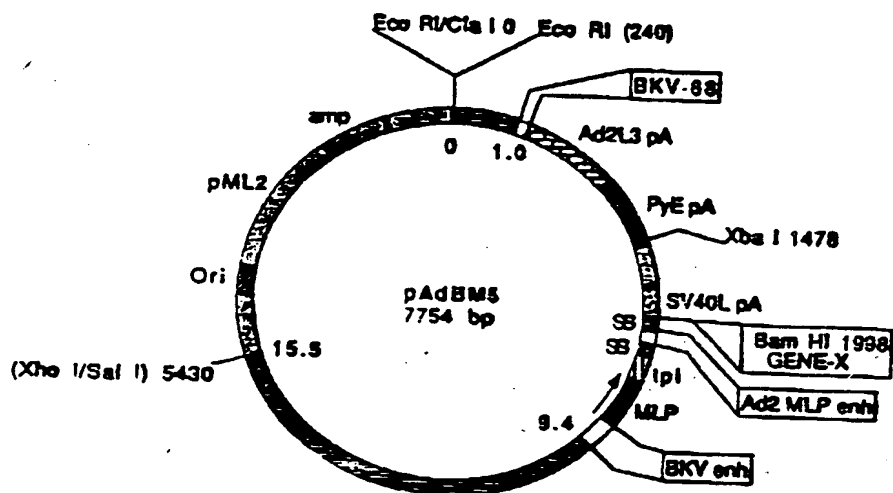
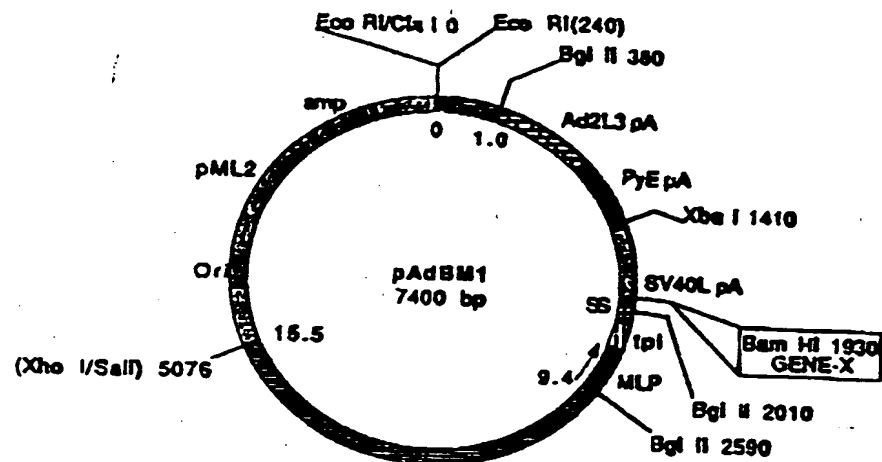
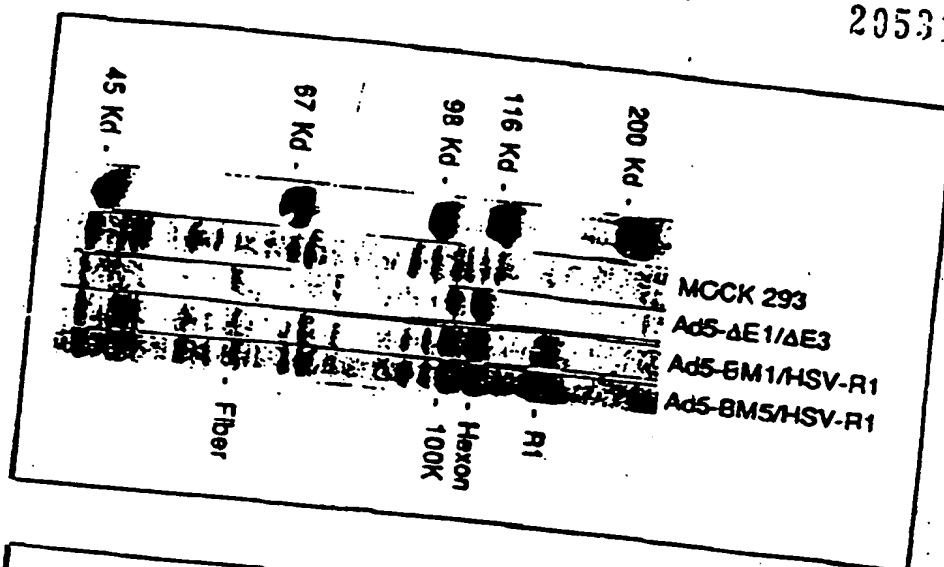


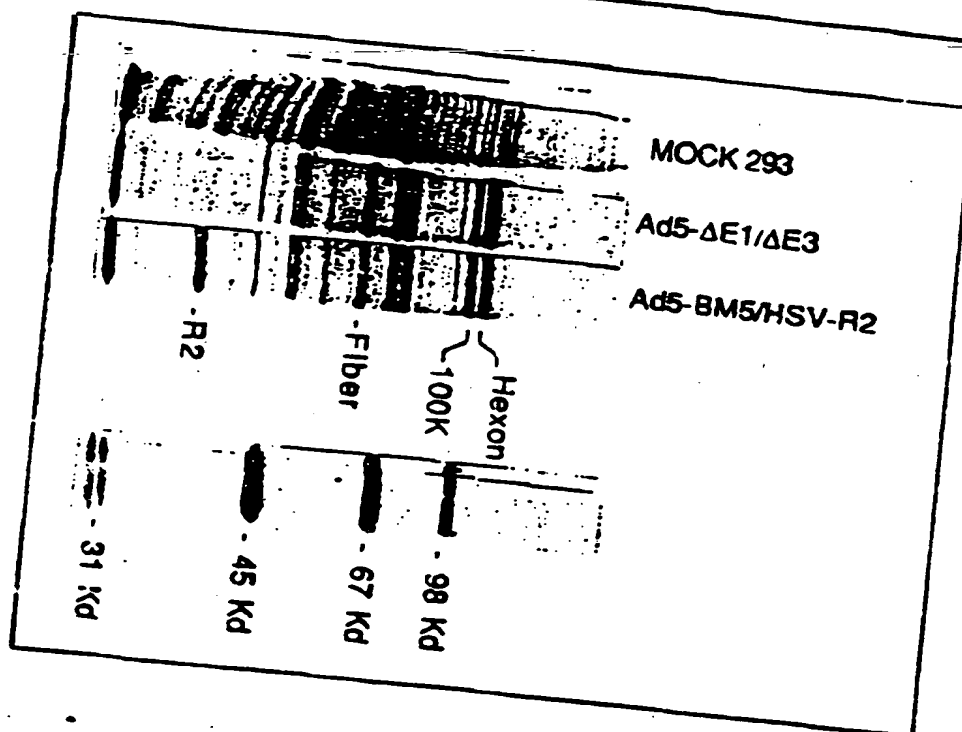
Fig 5

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A

3.5



B

3.5

2.053187

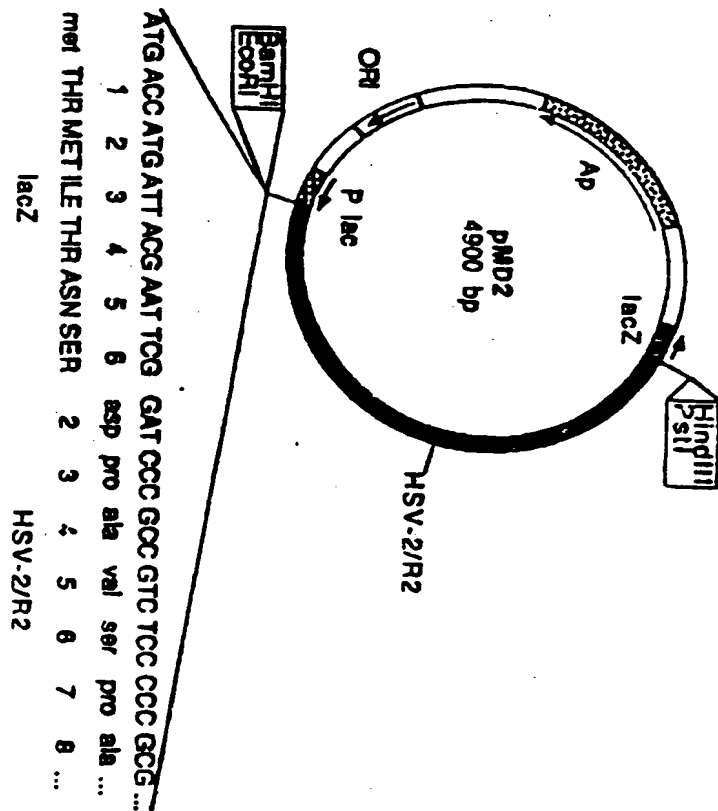
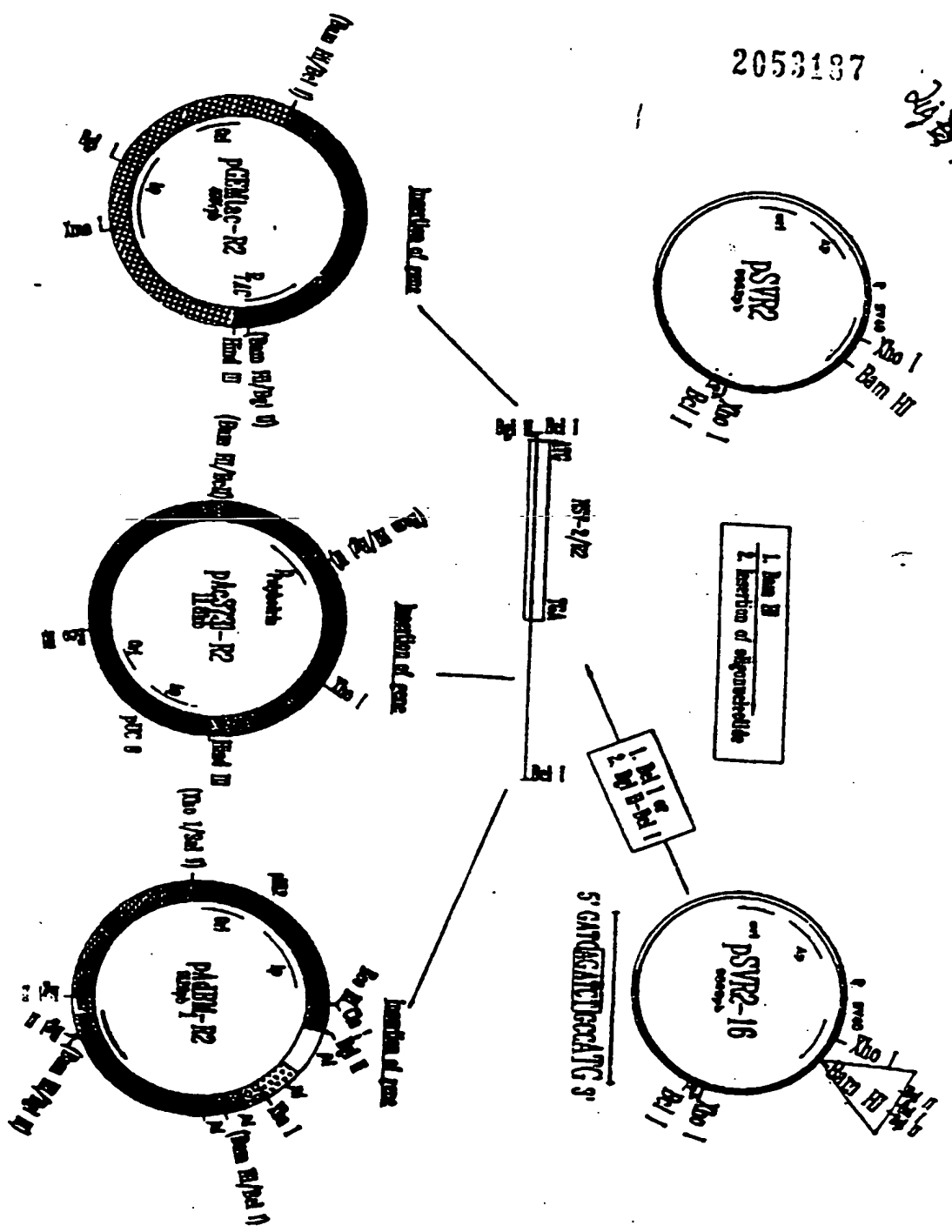


FIGURE 6



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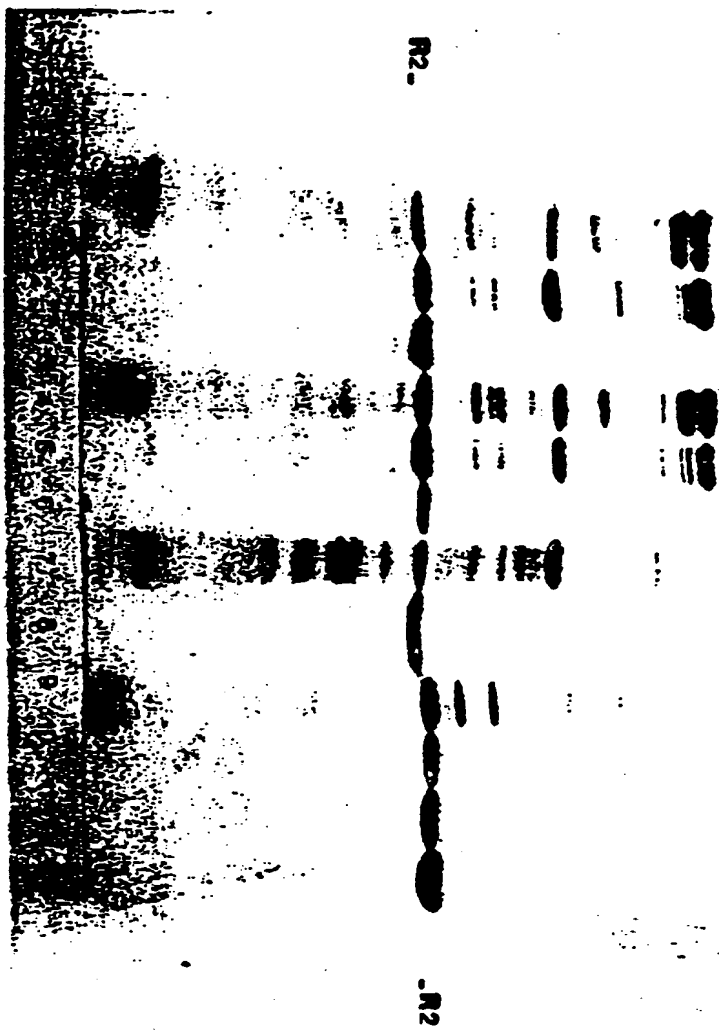


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<u>ADSSNR2</u>	<u>ADSSNR72</u>	<u>SACR2</u>	<u>PMS2</u>	%N2
4    14    60    8	20    57	36    21    90    99    99		
60    25    10    50    50    25    6    50    14    24    6    5    10				wg

→ often can measure PMS2.

To compare with above step of Bm1 and Bm5



**-R2**